Calcitonin Induces Hypertrophy and Proliferation of Pars Intermedia Cells of the Rat Pituitary Gland

TOSHI HORIUCHI, MITSUI ISOBE, MINORU SUZUKI
and YASUO KOBAYASHI[1]

*Safety Research Department, Teikoku Hormone Mfg. Co., Ltd.,
Shimosakunobe, Kawasaki 213 and [1]Department of
Biology, Faculty of Science, Okayama
University, Okayama, Japan*

ABSTRACT—The effects of subcutaneous administration of synthetic salmon calcitonin (sCT) on the pars intermedia (PI) cells of the pituitary gland in the rat were investigated histologically, immunohistochemically, and electron microscopically. Chronic administration of sCT at 0.75, 7.5, 30 or 120 IU/kg/day for 4 or 52 weeks to male and female rats induced hypertrophy of PI cells and thickening of the PI without any sign of toxicity. The mean size of PI cells was significantly increased in sCT-treated rats compared to vehicle treated rats, and the magnitude of increase was dose-dependent. sCT treatment of male rats at 120 IU/kg for 3, 7 and 14 days induced 2.8-, 2.6-, and 2.3-fold increase, respectively, in the rate of cell proliferation in the PI estimated by labeling of the nuclei with bromodeoxyuridine. Electron microscopic examination in male rats after sCT treatment at 120 IU/kg for 4 weeks disclosed Golgi complex hypertrophy with electron-dense secretory granules and expansion of rough endoplasmic reticulum. Immunohistochemical staining of the PI for α-melanocyte stimulating hormone and β-endorphin disclosed no marked differences between the control and sCT-treated rats. These results suggest that sCT at pharmacological doses stimulates and increases the thickness of the PI of the rat pituitary gland by increasing the rate of cell proliferation and by inducing hypertrophy of individual cells.

INTRODUCTION

The pars intermedia (PI) of the murine pituitary gland contains several peptides cosynthesized in a common precursor, proopiomelanocortin (POMC). These include α-melanocyte stimulating hormone (αMSH), βMSH, γMSH, corticotropin-like intermediate peptide, γ-lipotropin, met-enkephalin and β-endorphin [8, 24]. In mammals, αMSH and β-endorphin play a role in the control of pigmentation and are thought to be involved in the regulation of the endocrine and cardiovascular systems and in central processes such as analgesia, attention, and arousal [13, 25]. The PI is known to be regulated primarily by dopaminergic neurons emanating from the arcuate nucleus of the hypothalamus and terminating directly on PI cells [3, 31]. Thus, pharmacological manipulation with dopaminergic drugs has been shown to elicit changes in the rat PI morphology, secretion of POMC-derived peptides, POMC mRNA content, and rate of cell proliferation [4, 6, 7, 9, 14].

Recently, we observed non-functional tumors in the pars distalis of the rat pituitary gland [26] following the administration for one year of synthetic salmon calcitonin (sCT), which lowers the serum calcium level by inhibiting bone resorption [28]. In these experiments, we also noted hypertrophy of the PI in the sCT-treated rats. To elucidate the effects of sCT on the cells in the rat PI, we histologically and immunohistochemically examined the cell size and the cell proliferation rate after subchronic and chronic treatment with sCT, using monoclonal antibody against bromodeoxyuridine (BrdU), together with ultrastructural observation.

MATERIALS AND METHODS

Six-week-old male and female Sprague-Dawley rats (Charles River Japan Inc., Atsugi) were used in the experiments. The rats were housed individually in plastic cages and given standard laboratory feed (CE-2, Clea Japan Inc., Tokyo) and tap water ad libitum. The rats were kept under room temperature and relative humidity conditions of $22\pm2°C$ and $60\pm10\%$, respectively, on a 12–12 hr light-dark cycle.

The sCT (Teikoku Hormone Co. Ltd., Kawasaki) is the type-I form of naturally produced salmon calcitonins [11], and has a potency of about 5000 IU/mg. The sCT was dissolved in an acetic buffer (pH 4.0). Haloperidol (Wako Pure Chemical, Tokyo) was suspended in saline solution containing 1% Tween-80, and BrdU (Sigma, St. Louis, USA) was dissolved in 50% dimethylsulfoxide solution.

In the first experiment, the effects of long-term administration of sCT at various doses on the size and morphology of PI cells were examined. This experiment was carried out as a part of a one-year chronic toxicity study of sCT. Groups of 10 rats of each sex were injected subcutaneously with vehicle or with sCT at 0.75, 7.5, 30 or 120 IU/kg once daily for 4 or 52 weeks. The rats were killed on the day after the last injection under ether anesthesia. The pituitary glands were removed, fixed in 10% buffered formalin, and embedded in paraffin. Sections 4 μm thick were cut in the frontal plane and stained with hematoxylin and eosin. In the measurement of the size of PI cells, 3 sections were obtained from the mid-portion of each PI. On the light micrographs of these sections, the number of nuclei of PI cells was counted, and the area occupied by these cells was measured. Finally, for each PI, the size of a single cell was calculated by dividing

the total area by the number of nuclei. Immunohistochemical staining for αMSH and β-endorphin was carried out on sections of the pituitary from 5 male rats in each group treated with vehicle or sCT (120 IU/kg) for 4 or 52 weeks by the avidin-biotin-peroxidase complex (ABC) method [15], using a Nichirei ABC Kit (Nichirei, Tokyo). The antibodies against αMSH (dilution, 1:2000) and β-endorphin (1:4000) were obtained from UCB-Bioproducts, Belgium. For the ultrastructural observation, the pituitary glands of 5 male rats treated with vehicle or sCT (120 IU/kg) for 4 weeks were fixed in 2.5% glutaraldehyde for 3 hr at 4°C, followed by 1% OsO_4 for 1 hr, and then embedded in Quetol 812. Ultrathin sections were stained with uranyl acetate and lead citrate, and examined with a JEOL-1200EX electron microscope.

In the second experiment, the effect of sCT on the PI cell proliferation was estimated by the BrdU immunohistochemical technique described by Tatematsu et al. [30]. In this study, before the rat was killed, continuous administration of BrdU (120 μg/hr) into the peritoneal cavity was carried out for 72 hr through Alzet osmotic minipumps (Model 2001, Alza Corp.,Palo Alto, Calif., USA). The superior sensitivity of continuous labeling in comparison with pulse labeling for assessment of DNA replication in rodent tissues has been well documented [10]. Groups of 15 male rats each received daily administration of vehicle or sCT (120 IU/kg) by subcutaneous injection or haloperidol (10 mg/kg) orally once daily for 3, 7 or 14 days. Five rats in each group were sacrificed on days 3, 7 and 14. The pituitary glands were fixed in Bouin's solution for 4 hr, embedded in paraffin, and sectioned in the frontal plane. Sections 4 μm thick were hydrolyzed with 2N HCl for 20 min at 25°C and neutralized with 0.1 M boric acid-borate buffer (pH 7.6) for 10 min at 25°C, and then digested for 3 min at 37°C with 0.04% actinase (Kaken Kagaku, Tokyo) in 0.01 M phosphate-buffered saline. The monoclonal antibody of BrdU (Becton-Dickinson, Mountain View, CA, USA; dilution, 1:100) was used for immunohistochemical staining using the Nichirei ABC-Kit. After immunostaining, the sections were counterstained with hematoxylin. The BrdU labeling index was defined as the percentage of BrdU-labeled cells per total cells (more than 1000 PI cells) in 2 sections of each PI.

Data are expressed as mean±SD. Statistical analyses were conducted with multiple comparison test after one-way analysis of variance (ANOVA) of the data for the PI cell area and Student's t-test for the BrdU labeling index.

RESULTS

PI histology and mean cell size

In the vehicle-treated rats, the PI was composed of 10 or more layers of closely packed cells divided into lobules by strands of connective tissue. The principal type of PI cells was a polyhedral cell with an ovoid smooth nucleus (Fig. 1a). A small number of interstitial cells were also distributed throughout the parenchyma of the PI.

Chronic sCT treatment for 4 or 52 weeks induced hypertrophy of the PI cells in both male and female rats (Fig. 1b). sCT treatment also increased the thickness of the PI without inducing any disorder of the lobular structure or degeneration of PI cells. Compared with that in the vehicle-treated rats, the mean cell size was significantly increased in the male rats treated with sCT at the doses of 7.5 IU/kg or more and in the female rats treated with sCT at the doses of 30 and 120 IU/kg (Table 1). The magnitude of this increase was dose-dependent.

TABLE 1. The mean PI cell area in male and female rats treated subcutaneously with sCT for 4 or 52 weeks

sCT (IU/kg/day)	Mean PI cell area±SD (μm^2)			
	4 weeks		52 weeks	
Male				
0	(10)[a]	131.3±14.3	(10)	116.3± 9.9
0.75	(10)	141.5±18.0	(10)	124.7±11.9
7.5	(10)	155.2±18.6*	(8)	136.2±23.8
30	(10)	178.8±17.6**	(5)	160.2±26.2
120	(10)	187.5±18.0**	(6)	200.7±21.5**
Female				
0	(10)	110.0± 6.0	(10)	105.4±10.3
0.75	(10)	107.7± 8.6	(10)	105.6± 7.0
7.5	(10)	113.7± 8.6	(10)	109.4±11.1
30	(10)	126.0±11.5**	(10)	156.1± 9.7**
120	(10)	151.0±14.1**	(10)	186.4±12.3**

[a] No. of rats.
* p<0.05.
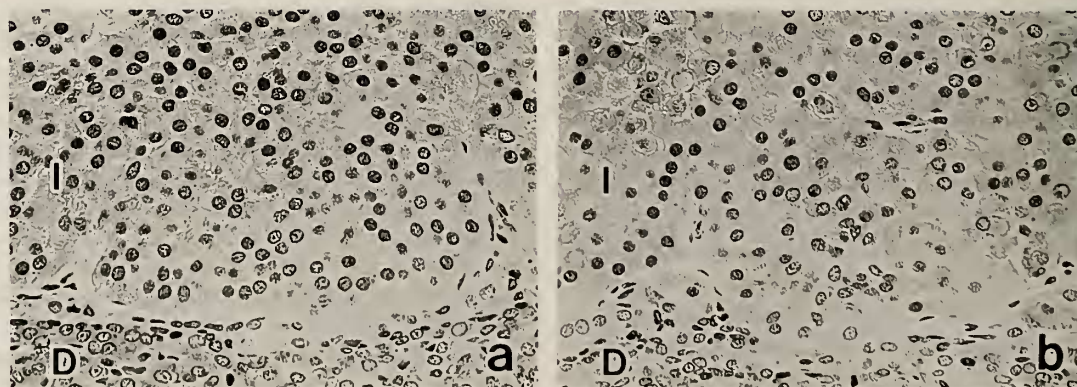** p<0.01 vs. vehicle control.

FIG. 1. Light micrographs of the PI of the pituitary gland in male rats treated subcutaneously with vehicle (a) or 120 IU/kg of sCT (b) for 4 weeks. The PI cells in the rat given sCT demonstrate diffuse hypertrophy. I, pars intermedia; D, pars distalis. Hematoxylin-eosin, × 150.
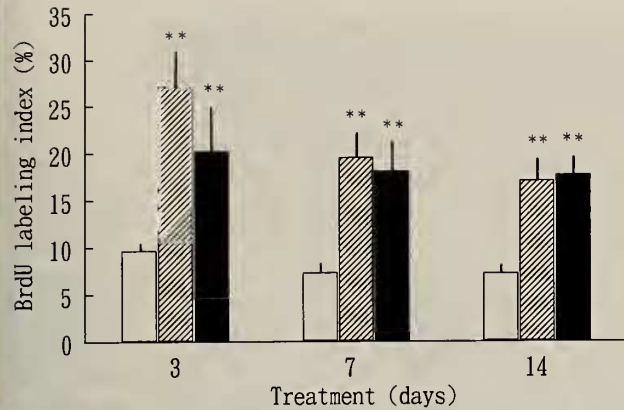
FIG. 2. Effect of repeated sCT or haloperidol treatment on BrdU labeling index in the PI of the rat pituitary gland. Groups of five rats each were treated with vehicle (s.c.), 120 IU/kg of sCT (s.c.), or 10 mg/kg of haloperidol (p.o.) for 3, 7 and 14 days. BrdU (120 μg/hr) was injected continuously through an osmotic minipump for 72 hr prior to sacrifice. Data are expressed as mean ± SD. Open columns, vehicle-treated; hatched columns, sCT-treated; closed columns, haloperidol-treated. **, $p < 0.01$ vs. vehicle controls.

Cell proliferation in PI

After the treatment for 3, 7, and 14 days, the mean BrdU labeling indices in the sCT-treated rats were significantly higher (2.8-, 2.6-, and 2.3-fold, respectively) than those in the rats given vehicle only (Figs. 2 and 3). Haloperidol also significantly increased the number of cells labeled by BrdU (more than 2-fold) after all 3 treatment periods.

Ultrastructural findings of PI

In the vehicle-treated rats, the glandular cells of the PI contained numerous secretory granules of variable density (Fig. 4). Secretory granules were distributed randomly throughout the cytoplasm. The Golgi complexes, with a small number of electron-dense granules, were rather inconspicuous (Fig. 4). The rough endoplasmic reticulum was scarce.

The PI cells in the sCT-treated rats were highly enlarged, and the secretory glanules were located at the periphery of the cells. The Golgi complexes showed marked hypertrophy associated with an increase in the number of electron-dense
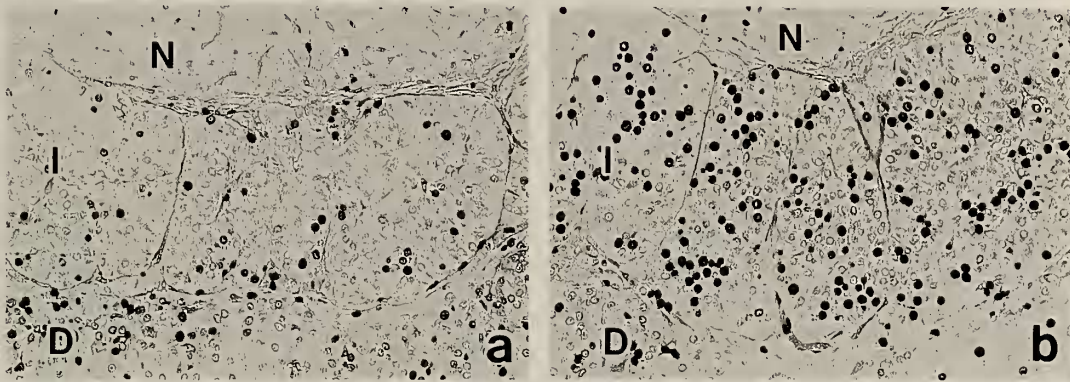

FIG. 3. Immunohistochemistry of BrdU in the pituitary PI of male rats treated with vehicle (a) or 120 IU/kg of sCT (b) for 3 days. The dark nuclei are BrdU-labeled nuclei. The sCT treatment markedly increased the number of BrdU-positive cells. I, pars intermedia; D, pars distalis; N, pars nervosa. ×130.
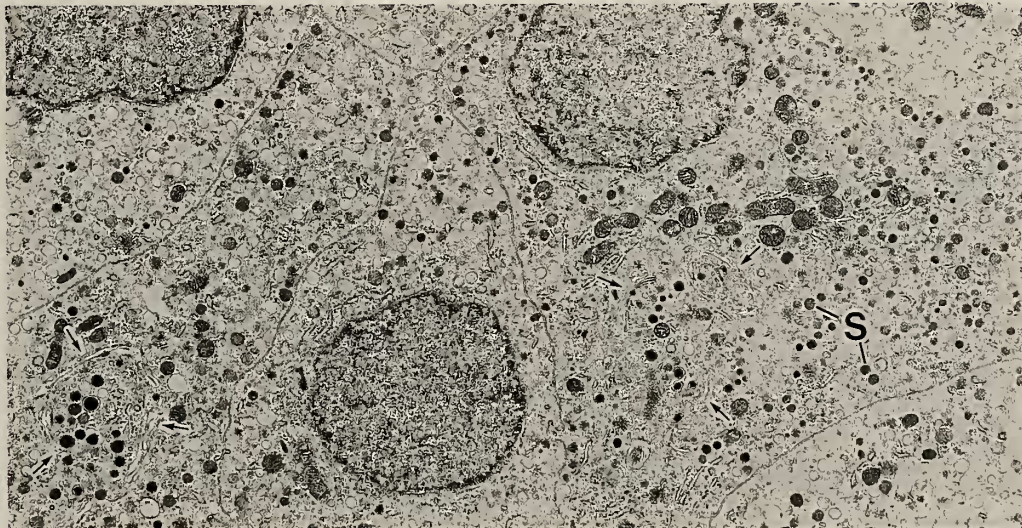

FIG. 4. Electron micrograph of the PI cells in a male rat treated with vehicle for 4 weeks. The cytoplasm contains numerous secretory granules (S) and Golgi complexes (arrows) with a small number of electron dense granules. ×6500.
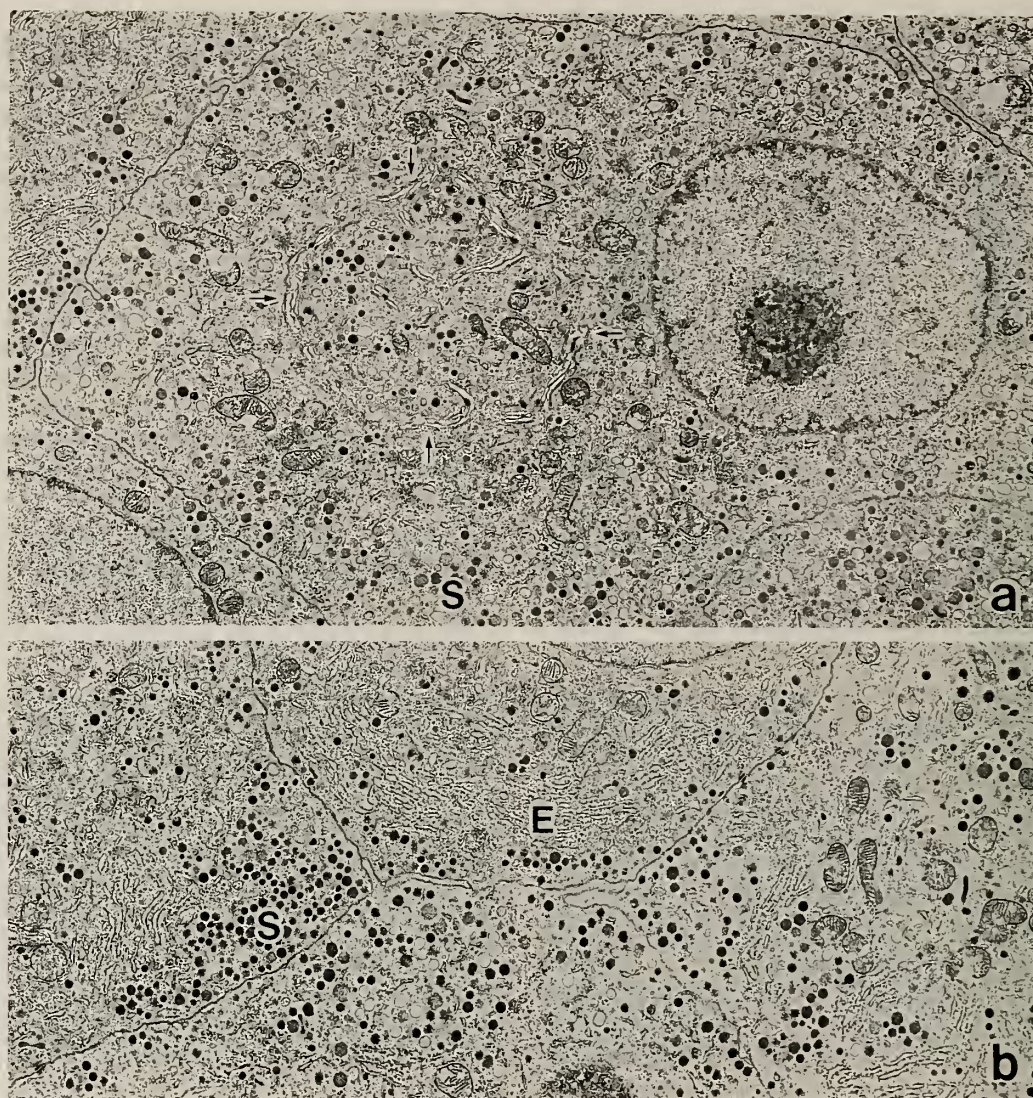
FIG. 5. Electron micrographs of the PI cells in a male rat treated with 120 IU/kg of sCT for 4 weeks. Note the activated Golgi complex (arrows), accumulation of secretory granules (S) in the cell periphery (a), and well-developed rough endoplasmic reticulum (b). E, rough endoplasmic reticulum. ×6500.
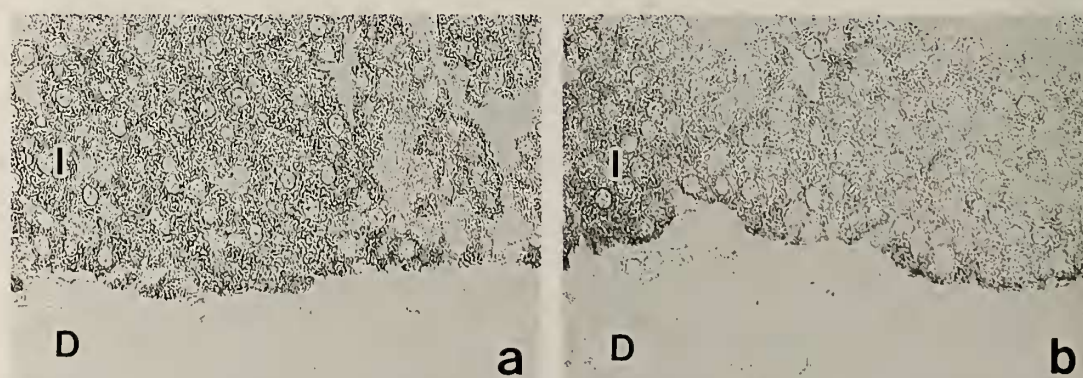

FIG. 6. Immunohistochemistry for αMSH (a) or β-endorphin (b) in the pituitary PI of male rats treated with 120 IU/kg of sCT for 4 weeks. Except for interstitial cells and cleft cells, all PI cells are equally stained by antibodies against αMSH and β-endorphin. I, pars intermedia; D, pars distalis. ×165.

secretory granules (Fig. 5a). The rough endoplasmic reticulum was conspicuous and well developed, and many of the PI cells showed parallel arrays of rough endoplasmic reticulum (Fig. 5b).

Immunohistochemistry for α-MSH and β-endorphin

All PI cells were equally stained by antibodies against αMSH and β-endorphin in both vehicle- and sCT-treated rats excepting the cells of the interlobular connective tissue, interstitial cells, and marginal cells lining Rathke's cleft (Figs. 6a and b). A small number of cells in the pars distalis were also stained by antibodies against αMSH and β-endorphin. These immunohistochemical staining features were identical to those described by Kurosumi *et al.* [22]. No difference was seen between the control and sCT-treated rats in the immunostaining for αMSH and β-endorphin.

DISCUSSION

Chronic treatment of rats with sCT was shown to induce hypertrophy of PI cells and increased thickness of the PI without any sign of toxicity or alteration of immunostaining for αMSH and β-endorphin. The size of PI cells showed dose-dependent increase in the sCT-treated rats. Electron microscopic examination revealed that sCT treatment caused hypertrophy of Golgi complexes associated with an increase of electron-dense secretory granules and expansion of rough endoplasmic reticulum. Such cytological changes have been observed in dark-adapted amphibians [27], which are known to release MSH under that condition [12]. In mammals, the PI cells also exhibit changes similar to those described herein under a variety of conditions or during drug treatment which enhance the activity of the PI, such as exposure to bright light and loud noises [23], variation in hydromineral intake [17–21], and reserpine, metapirone and haloperidol injection [7, 29, 32]. Thus, the morphological changes after sCT treatment strongly suggest that sCT enhances the activity of the PI of the rat pituitary gland.

The increase in the rate of cell proliferation of the PI is further evidence that the PI is stimulated by sCT treatment. The daily administration of sCT for 3, 7 and 14 days produced 2.8-, 2.6-, and 2.3-fold increase, respectively, in the rate of cell proliferation estimated by BrdU labeling. This increase after sCT treatment is indicative of hyperfunction of the PI, since haloperidol, a D-2 dopamine receptor antagonist which enhances PI function, increases and bromocriptin, a D-2 agonist which inhibits the PI, decreases the rates of cell proliferation as determined by the uptake of [³H]thymidine or by the mitotic index [7]. In the present experiment, haloperidol also produced more than a 2-fold increase in the rate of cell proliferation in our model.

It has been shown that sCT administration to rats markedly increases the urine water and sodium excretion and causes hyponatremia [1, 16]. The diuretic and natriuretic activities of sCT were confirmed in the present studies (data not shown), and the sCT-treated rats developed pronounced polyuria within a few days. Although the mechanism is poorly understood, there is considerable evidence for the involvement of the PI in hydromineral regulation through the control of aldosterone secretion by the adrenal cortex [5, 17, 20]. Therefore, variation in salt intake alters the function and morphology of the PI. Dehydration selectively activates the tuberohypophyseal dopaminergic neuronal system and inhibits the PI in rats [2]. On the other hand, hyponatremia induced by dietary sodium deprivation [17, 18, 21] as well as excessive water intake induced by food deprivation and concurrent free access to glucose solution [19], conditions exactly the opposite to dehydration, enhance the activity of PI cells. The physiological state induced by sCT treatment is similar to a condition opposite to dehydration. Thus, there is a posibility that alteration of hydromineral metabolism by sCT attenuates the dopaminergic inhibitory control and stimulates the PI cells. Further biochemical studies and urinalyses in relation to the function of the adrenal zona glomerulosa are in progress to examine the mechanism of activation of the PI in rats after sCT treatment.

ACKNOWLEDGMENTS

REFERENCES

1 Aldred JP, Kleszynski RR, Bastion JW (1970) Effects of acute administration of porcine and salmon calcitonin on urine electrolyte excretion in rats. Proc Soc Exp Biol Med 134: 1175–1180

2 Alper RH, Demarest KT, Moore KE (1980) Dehydration selectively increases dopamine synthesis in tuberohypophyseal dopaminergic neurons. Neuroendocrinology 31: 112–115

3 Baumgarten HG, Björklund A, Holstein AF, Nobin A (1972) Organization and ultrastructural identification of catecholamine nerve terminals in the neural lobe and pars intermedia of the rat pituitary. Z Zellforsch 126: 483–517

4 Beaulieu M, Goldman ME, Miyazaki K, Frey EA, Eskay RL, Kebabian JW, Cote TE (1984) Bromocriptine-induced changes in the biochemistry, physiology, and histology of the intermediate lobe of the rat pituitary gland. Endocrinology 114: 1871–1884

5 Brownie AC, Pedersen RC (1986) Control of aldosterone secretion by pituitary hormones. J Hypertention 4 (Suppl 5): s72–s75

6 Chen CLC, Dionne FT, Roberts JL (1983) Regulation of the pro-opiomelanocortin mRNA levels in rat pituitary by dopaminergic compounds. Proc Natl Acad Sci USA 80: 2211–2215

7 Chronwall BM, Millington WR, Griffin WST, Unnerstall JR, O'Donohue TL (1987) Histological evaluation of the dopaminergic regulation of proopiomelanocortin gene expression in the intermediate lobe of the rat pituitary, involving *in situ* hybrization and [³H]thymidine uptake measurement. Endocrinology 120: 1201–1211

8 Eiper BA, Mains RE (1980) Structure and biosythesis of pro-adrenocorticotropin/endorphin and related peptides. Endocr Rev 1: 1–27

9 Farah JM, Malcolm DS, Mueller GP (1982) Dopaminergic inhibition of pituitary β-endorphin-like immunoreactivity secretion in the rat. Endocrinology 110: 657–659

10 Goldsworthy TL, Morgan KT, Popp JA, Butterworth BE (1991) Guidelines for measuring chemically induced cell proliferation in specific rodent target organs. In "Chemically Induced Cell Proliferation: Implication for Risk Assessment Vol 369" Ed by BE Butterworth, TJ Staga, W Farland, M McClain, Wiley-Liss Inc, New York, pp 253–284

11 Guttmann S (1981) Chemistry and structure-activity relationship of natural and synthetic calcitonins. In "Calcitonin-Chemistry, Physiology, Pharmacology and Clinical Aspects" Ed by A Pecile, Excerpta Medica, Amsterdam, pp 11–24

12 Hadley ME, Bagnara JT (1975) Regulation of release and mechanism of action of MSH. Am Zool 15 (Suppl 1): 81–104

13 Hirsch MD, O'Donohue TL (1986) Structural Modifications of proopiomelanocortin-derived peptides markedly alter their behabioral effects. J Pharmacol Exp Ther 237: 378–385

14 Höllt V, Bergmann M (1982) Effects of acute and chronic haloperidol treatment on the concentrations of immunoreactive β-endorphin in plasma, pituitary and brain of rats. Neuropharmacology 21: 147–154

15 Hsu SM, Raine L, Fanger H (1981) Use of avidine-biotin-peroxidase complex (ABC) in immunoperoxidase techniques: A comparsion between ABC and unlabeled antibody (PAP) procedures. J Histochem Cytochem 29: 577–580

16 Keeler R, Walker V, Copp DH (1970) Natriuretic and diuretic effects of salmon calcitonin in rats. Can J Physiol Pharmacol 48: 838–841

17 Kobayashi Y (1974) Quantitative and electron microscopic studies on the pars intermedia of the hypophysis. Alterations of the pars intermedia and the adrenal zona glomerulosa of albino mice following sodium restriction. Annot Zool Jap 47: 221–231

18 Kobayashi Y (1974) Quantitative and electron microscopic studies on the pars intermedia of the hypophysis. Effect of short-term administration of a sodium deficient diet on the pars intermedia of mice. Cell Tissue Res 154: 321–327

19 Kobayashi Y, Kumazawa T, Takeuchi M (1984) A new method for inducing copious drinking and the accompanying stimulation on the pars intermedia of the mouse pituitary gland. Arch Histol Jap 47: 71–77

20 Kobayashi Y, Okada M (1990) Urea stimulation of pituitary pars intermedia cells of suckling under copious drinking. Zool Sci 7: 281–286

21 Kobayashi Y, Takema M (1976) A morphometric study on the pars intermedia of the hypophysis during impairment of the renin-angiotensin-aldosterone system in sodium depleted mice. Cell Tissue Res 168: 153–159

22 Kurosumi K, Tanaka S, Taniguchi Y, Tokuyasu K (1986) Differential subcellular localization of ACTH and related peptides in rat anterior pituitary corticotrophs. In "Pars Distalis of the Pituitary Gland-Structure, Function and Regulation" Ed by F Yoshimura, A Gorbman, Excerpta Medica, Amsterdam, pp 163–169

23 Moriarty GC, Halmi NS, Moriarty CM (1975) The effect of stress on the cytology and immunohistochemistry of pars intermedia cells in the rat pituitary. Endocrinology 96: 1426–1436

24 Nakanishi S, Inoue A, Kita T, Nakamura M, Chang AC, Cohen SN, Numa S (1979) Nucleotide sequence of cloned cDNA for bovine corticotropin-beta-lipotropin precursor. Nature 278: 423–427

25 O'Donohue TL, Dorsa DM (1982) The opiomelanotropinergic neuronal and endocrine systems. Peptides 3: 353–395

26 Osamura RY, Murakoshi M, Inada R, Horiuchi T, Watanabe K (1992) Biological aspects of pituitary turmors induced by synthetic salmon calcitonin (TZ-CT) in Sprague-Dawley rats. Acta Pathol Jpn 42: 401–407

27 Perryman EK (1974) Fine structure of the secretory activity of the pars intermedia of Rana pipiens. Gen Comp Endocrinol 23: 94–110

28 Raisz LG, Au WYW, Friedman J, Niemann I (1967) Thyrocalcitonin and bone resorption. Studies employing a tissue culture bioassay. Am J Med 43: 684–690

29 Saland LC (1978) Effects of reserpine administration on the fine structure of the rat pars intermedia. Cell Tissue Res 194: 115–123

30 Tatematsu M, Fukushima S, Aoki T, Mera Y, Inoue T, Ito N (1987) Patterns of epithelial proliferation revealed by continuous administration of bromodeoxyuridine during urinary bladder carcinogenesis in rats. Jpn J Cancer Res 78: 879–882

31 Tilders FJH, Smelic PG (1977) Direct neural control of MSH secretion in mammals: The involvement of dopaminergic tuberohypophysial neurons. Front Hormone Res 4: 80–93

32 Weman B, Nobin A (1973) The pars intermedia of the mink, Mustela vison. Fluorescence, light and electron microscopical studies. Z Zellforsh 143: 313–327